Optical Spectroscopy and Microscopy
Prof. Balaji Jayaprakash
Centre for Neuroscience
Indian Institute of Science - Bangalore

Lecture 43
Fundamentals of Optical Measurements and Instrumentation

Hello and welcome to this lecture series on the Spectroscopy and Optical Spectroscopy and Microscopy. We are in the devices section of this course and what we have seen in the previous lecture is, how do we go about setting up our confocal detection, in doing so I said we need to use those two special kinds of optical elements dichroic and the emission filters. These are just the variations of general class of filters that we have seen in the course, the interference based filters.

It can also be absorption based filters, but typically when in a fluorescent detection, you are trying to maximize your efficiency of discerning out the fluorescence photon from the excitation or the scattered photons. So typically you use the interference filters. They offer you a high selectivity per mm thickness of the filter that you need to put in. So you tend to go for the interference filters.

While the principle of operation is discussed in the class in terms of the theory of what happens to the light, when it passes through this small coating, the coating of organic or inorganic material on top of this transparent optics. When you do that, you we know that the depending on the thickness of these coatings, you can have the constructive or destructive interference happening. This is clearly dependent on the wavelength.

So allowing a particular wavelength to go through; while the others to get reflected. So now this can be put to use in this situation very nicely. So let us first take a look at how an emission filter itself, we would like to choose. So the idea here is to be able to clearly transmit most of or in fact all of, an ideal emission filter would really transmit every single fluorescent photon that your fluorophore is emitting.

While; at the same time blocking out every single excitation photon that gets scattered from uncollected back into the apparatus. Now if you just remember, if you go back to our setup that we have seen in the last class.

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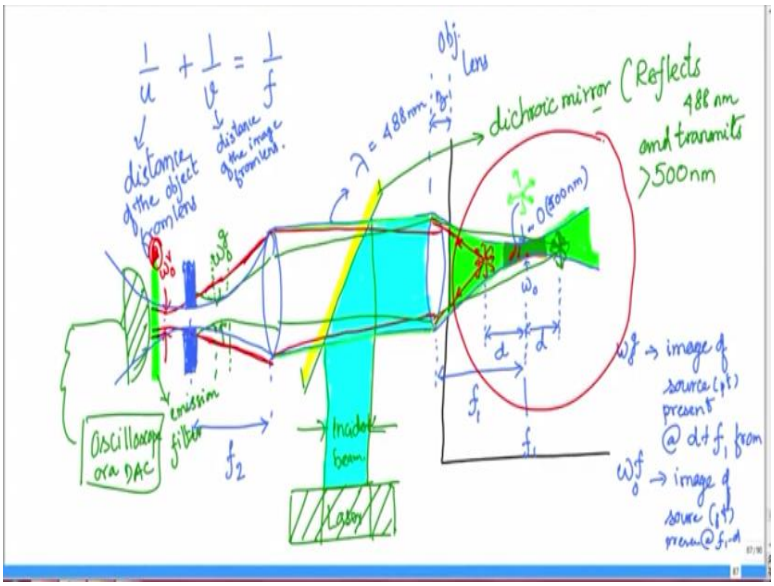

What you will see is that there is a fluorophore. This region if you actually look at, so this region let us say I am going to mark it out. So our sample is present here and your fluorescence is originating from this place that fluorescence gets collected by the detector. To generate that fluorescence we are actually taking in the laser beam and then focusing it with lens and so this laser beam actually gets reflected by the dichroic mirror and that focuses into the sample and the fluorescent gets passed through.

So when that happens not only the fluorescent photons and some amount of the backscattered excitation light also gets passed through, majority of them gets reflected just the way the incident beam is getting reflected , the back scattered photons also get reflected back onto the laser. While this on top of all of this, there is still leak-through of this back scattered excitation beam that comes in here.

Apart from that, there are scattered background lights that can go into the photomultiplier tube. Now what you want to do is, your emission filter that is located here you want this emission filter to be able to transmit only the fluorescence and the fluorescent photons and not the excited

or the backscattered light. So how do we do that? So let us take a look at the transmission spectrum of one off such this device, which is the emission filter itself.

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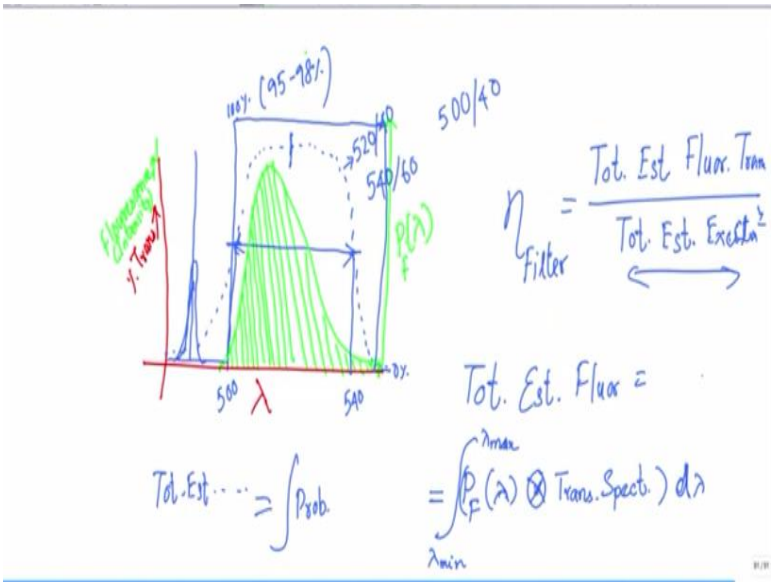

And I said the ideal transmission detector would be, if I were to plot, the spectrum like the here I am going to measure the transmission as a function of the wavelength. So let us plot out our emission itself okay. So let us say the emission is of the shape something like this and now what we would like to have in terms of, so the transmission here does not make sense, so here we will come, for plotting out the emission.

So we will say that either the fluorescence, fluorescence intensity okay and this is the percentage. I mean we are going to plot either the fluorescence intensity or the percentage transmission. In here, we are going to plot the fluorescence. So when we plot this it is important to pay attention to the Y-axis here. So what we are doing is that as a function of wavelength, we are actually telling, what is the probability of me finding the photon.

The way I have plotted is not necessarily the probability, when you are plotting the intensity, it actually gives you in a spectrometer what you will get is typically some digitisation units, each of this number in here right correspond to different amount of photons that are getting collected from here. So what we could do and one need to do to convert into a probability distribution is to actually measure this area, which is basically you add up all of your, okay.

You measure this area or you add up all the intensities in this wavelength range and then you basically area normalize the fluorescence. When your area normalizes the fluorescence, what you are asking is that if; there were to be one fluorescent photon that is emitted, now what is my probability that this photon will be having a wavelength right. In this axis, I am just actually plotting the probability P of lambda okay and of the fluorophore that I am actually interested in.

So what I am doing, what I am asking is that what is the probability that my given fluorescent photon will be in one of this wavelength okay. It is critical to make some estimates. We will use this in a little bit. So once you have that an ideal emission filter should be such that the transmission if you were to look at it, it does not transmit any light, particularly no. It does not transmit any of the light that is of lesser wavelength or a higher frequency to that of the fluorescence because your excitation light is somewhere here.

And ideally transmits every single fluorescent photon okay just at the start of it, it should go to 100% transmission and stay there until this entire range and then comes back down to 0% okay. So but that never happens, the reason being that it is a very hard to generate such sharp transitioning filters. So what you will see is that one is that there is no start transition and it is also hard to have a 100% transmission right. It is always there is some amount of reflection loss.

So what you will see is that you typically have somewhere between 95-98% transmission and since the transitions are not sharp and then the percentage transmission is 95-98 and the real world profile would look in this same scale, pretty much like that. So typically you will have a choice of a quite a few emission filters, right choice here is given by two factors. One at which point is this transition happening.

So since they are not a very sharply defined transition, it is customary to define the transition point as where the transmission has come down to half the initial value okay. So whatever the wavelength that it corresponds to again the same way this, now this gap would be our transmission bandwidth okay of the filter and the center of this wavelength would be the name.

Say for example, if this were to be such that this wavelength is 500 and it goes all the way till let us say 540 and it is a 40 nanometer and the center then would be 520. So you would call this filter as 520 band pass filter, which works the slash 40 okay. So if you choose a filter and nowadays you can get pretty much any arbitrary wavelength that you choose. You could actually encounter a filter that is like say 540 slash say 60 or some other filter, say given by say 500 slash 40.

Now which of the filter would be an ideal filter to use and what determines this idea that you mean is the question that we would like to ask. The first thing that we want to optimize here is the total transmission output. At the same time, we are actually looking here for selectivity and not just maximizing the transmission, so which means you need to be able to minimize the amount of light that is the excitation light that is getting through.

So it is not just you want to have a maximum transmission of the fluorescence, but also you want to have a minimum transmission of the excitation light. So we will look at to optimize the total estimated fluorescence throughput or transmission divided by total estimated excitation right. You would like to maximize this. So that is the goal. So a filter with maximum, so let us call that as efficiency of the filter okay. The way you calculate this is pretty simple.

So let us look at one term by term. So the total estimated fluorescence, the way you do that is basically once you have the probability distribution of the area normalized fluorescence, then what you do is, you convolve the transmission profile of the filter that you are interested in with that of the area normalized fluorescence. So what it tells you is that now if you convolve, so let us say your probability distribution function of the emission spectrum is given by P of F of lambda.

What you do is, you convolve with the transmission spectrum of your emission of the other filter. You convolve with the emission filter, since we are dealing with emission filter so that goes without saying. So when you convolve, what is the meaning of control here? Convolve, practically what it means is that you can multiply, you have to match the wavelength, if you have to be properly sampled and all that stuff meaning, if you have not.

Then you have to fit it to a polynomial interpolate, so that their sampling is equal basically at every corresponding wavelength of your transmission filter or your fluorescence, you need to have the transmissions of the filter and if you do that you multiply and if you have that, then you multiply with each other. Now that would give you the probability that there will be an emitted fluorescence photon at a given wavelength that you are looking at lambda.

And that will also make it through the transmission okay. So now once you have this, what we are going to do is then we are going to integrate this between the range of the emission filter which is lambda minimum within the band region that we are interested in. We are going to integrate it from lambda minimum to lambda max okay d lambda. So now this number, once you integrate is nothing but your numerator for the efficiency of the filter which is given by total estimate blah, blah.

Now for measuring the denominator, which is the total estimated excitation light that goes through the filter what we would like to know is that see, it will come. When you are actually measuring the transmission, you will see. In a little while, I am going to show you the real-world example of how the spectrum of a transmission emission filter looks like, but if you look at it, you will see it is coming very close to about zero.

And zero transmission is of not much use, because zero to what our degree right, it is 0.000 or 0.1 or zero point and so on and so forth. So it is more sensitive in this case to know the optical density, that is OD and they are related 1 over T, as I guess you know, the point is that if you actually have the OD, measurement in OD, then even the smallest change in transmission, you will be able to put a number to it.

So we will have to look at this spectrum, the transmission spectrum not in percentage transmission, but in OD, optical densities to determine how much of the blockade to the excitation light this filter is offering and let us say if you are getting 5, I mean some x optical density blockade what it means is that, you are measuring the thing the transmission in the log scale, so for every OD, every optical density that you extract from the filter spectrum.

So you have to do the same thing for your excitation profile too. The lasers are typically pretty sharp and depending on the kind of laser that we are using, if you are using a diode laser, you have to be little careful where there is a slightly wider bandwidth okay. So it may be somewhat this. So we have to do the exact same thing what we have done for this. We have to represent this as in the probability function and then multiply by the transmission of the emission filter obtained by actually looking into the absorption spectrum okay blockade.

And then you actually calculate back the transmission from there and we will do that in an example as well as in terms of the real-world sample right now in the course. So when you do that, so then you take that and then plug it into the denominator here. So thereby you can actually determine the efficiency of this emission filter. Now this would be the case, if you are actually using just the emission filter.

So there could be a fantastic emission filter with a very good eta that the way we have defined, since they do not operate just all alone by themselves you need to use them in conjunction with a dichroic mirror.

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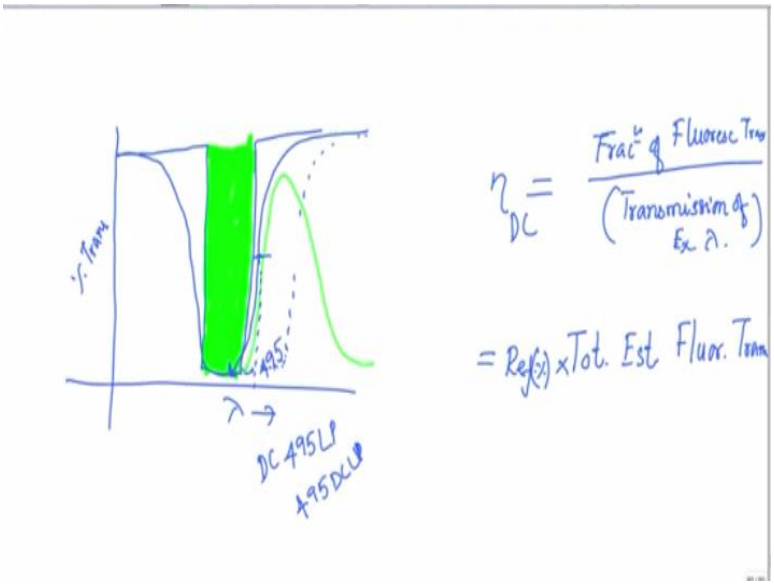

So a dichroic mirror the transmission would be, if you actually again do this. So we can actually do the percentage transmission. An ideal dichroic would be such that it undergoes, see there are

no such. Ideal dichroic will have a very sharp transmission. So it will remain like this and then I am sorry so right. So all the light that are present all the wavelength that are present in this region would get reflected by this dichroic.

So that is why, there is very low percentage of transmission, while rest everything before and after goes through okay, but the real world dichroic are nowhere near this and in fact what you would like to see is that I mean, what you often see is slow incoming region and then it goes. So these are actually mentioned by the halfway point, which I talked to you and told you about before, so about their lambda on their rising transition.

So in this case, let us say if this were to be at in our case, you would like to have the 488 nanometer being reflected. So let us say this can be going at about 495 or so at which, at 495 you want the 50% transmission, so that at 488 that is about here you really have a very good reflection. So you want to have this as close as possible. You do not want to have very wide, the reason being, because if you remember that the fluorescence itself the fluorescence spectra is redshifted.

But the redshift is only so much, so you do not want to cut into your fluorescence spectra as much as possible. So you see the fluorescence might be anywhere it here. So you do not want to be shifting your dichroic somewhere here. If the dichroic were to be here, you will see that much your fluorescence will get reflected to, you lose them. So you do not want that. So what you want to do is, I mean while on the other hand you in terms of the reflecting the maximum amount of excitation lamp.

So the wider you go, I mean the longer you go, I mean farther you go from your central wavelength, then the higher is the chance of you having a perfect reflection, because these transitions are not sharp. So that you have enough time for the transmission curve to slowly rise up; however, the optimization has to be kept in, for you to optimize you have to keep in mind you are not eating away the fluorescence.

So you want to be somewhere well before the onset of the fluorescence. So again we can actually in this case define eta of a dichroic filter. Before going there, the dichroic filters nomenclature is that we would pick the wavelength where we are actually rising by half. So it is a dichroic filter DC 495 longpass. So you can either call like this or 495 DCLP, so dichroic long pass filters. So they are named that way.

But then the efficiency here then what you want to do is that how do you want to define is that again the person that the fraction of fluorescence that is transmitted by here, what you actually want to do is that you want to have the maximum reflection right. So you want to have lower transmission okay for the excitation lambda. So now, if we write like this the important assumption is the fraction of the light that is not transmitted is getting reflected.

So this is going by the same logic as that of the emission filter. We are trying to be consistent with that. So the other way and a more useful way then is that the same thing as that a same numerator as that of this. So I am going to copy it. So we will have that right and what we will do is that we will multiply that by the reflection, the percentage reflection of the excited P okay. So you want to maximize this.

So when you are trying to choose the emission, I mean the dichroic and the filter, you want to optimize both of this. So the best way would be to actually estimate this number, which is consistent, I mean the definition is consistent between the dichroic and the filter and then optimize the product okay. You compare the products for different pairs, such that the pair with the maximum number or a maximum throughput will be the ideal pair. So just to illustrate this point, we are going to look at the real world example here.

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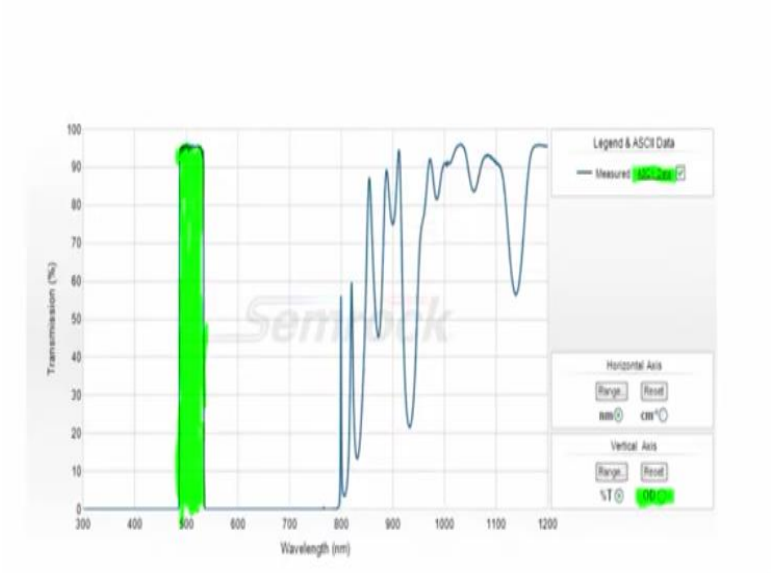

So I have picked up this emission filter and the dichroic spectrum. There are different companies, but I have picked up one, the Semrock and so now what we are actually looking at is the emission filter. So as you can see, actually this is for the 500, I mean 510 40 nanometer bandwidth, if I am not wrong. So you can see that is the portion that gets transmitted. Now if you go in there, if you know which company's emission filter you have.

All of the companies put out their emission spectrum online and then if you go in there, you can actually get this ASCII data. It is very useful, because the curves or the lines are nice to visualize but for you to estimate the eta that we have written down. You need to know the numbers right, for it corresponding to each of this wavelength that can be obtained by going and asking for the ASCII data and then, of course, if you notice here, you will also see either you can obtain this in terms of percentage transmissions or in OD.

So you need to get both. So the percentage transmission for estimating the fraction of the fluorescence that goes through this and percentage OD to estimate more precisely the amount of excitation beam that this filter lets through. So the spectrum of the emission filter is something like this and then while that of the dichroic, if you actually look at it is pretty close. So it is pretty close to an ideal dichroic, which you see except for that transition.

I mean the degree or the rate at which this transitions is happening, but it is pretty close in the sense that you have almost a flat region here and here. The first thing you notice either here as well as, as well as are in this is that there is this gap.

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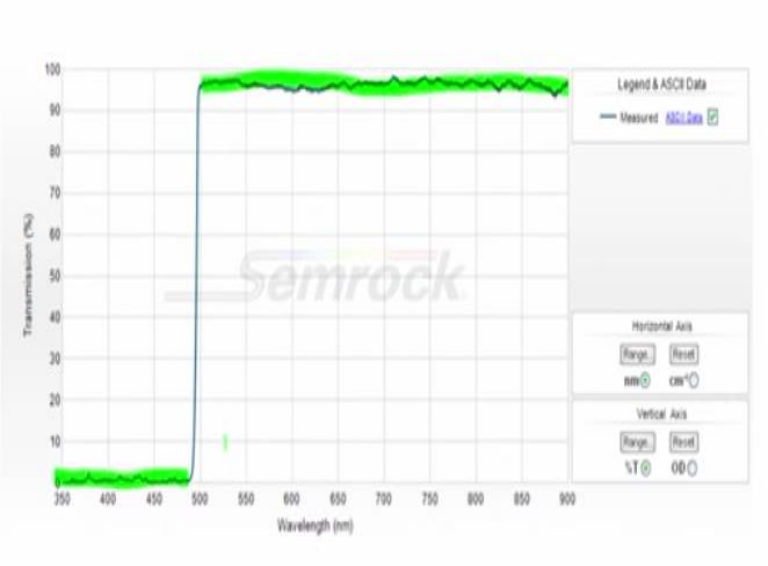

And as I was telling you, this not going 100%, it is about 95% or so and again the same things of obtaining the ASCII data and percentage transmissions or OD is here. So what you can actually do is that we will see it perhaps in the next class. If you plot out all of them together, you will be able to actually estimate this area, I mean, estimate the eta that we have defined.

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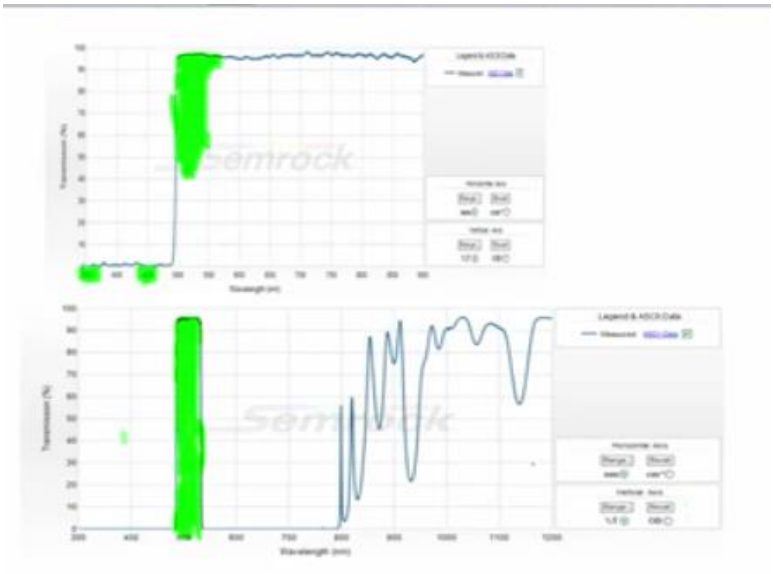

And for comparison I have also put both this spectra or on top of each other. Please note that the scales were different. So I have made it such that now it is 350 to 450, it is about 100 units is roughly about 100 nanometers and your coinciding with about 500 line here. So you will see pretty much 95% of the fluorescence that are originating in this band goes through. Actually we should start from here.

So 95% of the fluorescence originating in this band goes through this dichroic and of the 95% of the photons that are coming through another 95%, 95 of the 95% actually this emission filter lets through and so that is the percentage transmission of the system for the fluorescence. Similarly we can actually go ahead and estimate if you actually look at the spectrum in OD, what fraction of the light that the excitation beam actually comes through.

You will see that this filter is pretty good or optimized for detecting fluorescence originating from GFP which is centered around 500 millimeters. In the next class, we will actually put in this actual spectrums, overlay them all and then as we measure these numbers, we will compare them with 2 different filters and how they are stacking up to decide on the best filter. I would actually take example of YFP and a GFP and then see what suits the best, right. Thank you.